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Phenolic content of Sicilian virgin olive oils and their effect on MG-63 human osteoblastic cell proliferation

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SUMMARY: The aim of this study was, first, to investigate the influence of olive variety and elevation of orchards on the phenolic compound content of Sicilian virgin olive oils (VOOs) and, second, to investigate the effects of VOO phenolic extracts on osteoblast cell growth using the human MG-63 osteosarcoma cell line. Olive oil phenolic content and its effect on human osteosarcoma cell proliferation varied according to the type of cultivar and the grove altitude. This variation was also observed within the same type of cultivar. This observation demonstrates that the cultivar and the grove location can significantly affect the chemical composition and bioactivity of virgin olive oil. Although this study supports the hypothesis that virgin olive oil phenolic fractions exert a beneficial effect on bone health, further studies assessing the *in vivo* accessibility of virgin olive oil phenolic compounds to osteoblast cells should be carried out.

KEYWORDS: Osteoblastic cells; Phenolic compounds; Proliferation; Sicily; Virgin olive oil

RESUMEN: *Contenido fenólico de aceites de oliva vírgenes de Sicilia y su efecto sobre la proliferación celular de osteoblastos humanos MG-63.* El objetivo del presente trabajo es investigar la influencia de la variedad y la altitud del cultivo en el contenido fenólico de aceites de oliva virgen Sicilianos. Asimismo, se ha investigado el efecto de los extractos fenólicos de los aceites en el crecimiento de osteoblastos usando la línea celular de osteosarcoma humano MG-63. El contenido fenólico y el efecto de los extractos analizados en la proliferación de la línea celular osteoblástica muestra una variabilidad consistente de acuerdo con el tipo y la altitud del cultivo. Estos datos demuestran que estas características pueden afectar significativamente la composición química y los efectos en salud del aceite de oliva virgen. Los resultados de este trabajo soportan la hipótesis de que las fracciones fenólicas de los aceites de oliva vírgenes ejercen un efecto beneficioso en la salud ósea. Asimismo, se deben realizar más estudios que establezcan la accesibilidad *in vivo* de los compuestos fenólicos del aceite de oliva virgen a las células osteoblásticas.

PALABRAS CLAVE: Aceite de oliva virgen; Células osteoblásticas; Compuestos fenólicos; Proliferación; Sicilia

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1. INTRODUCTION

The olive tree is one of the most important crops in Mediterranean countries, especially in Spain, Greece, Tunisia and Italy (Baccouri *et al.*, 2007). Sicily has a long history of olive cultivation and is an important olive producing area (Poiana and Romeo, 2006). The large size of the island and its particularly fertile soil, which in Eastern regions is volcanic, allows for olive cultivation in many microclimates. Olives grown in the volcanic soil on the slopes of Mount Etna absorb minerals from the soil that subsequently produce a characteristic oil variety.

Considerable research has focused on the biologically active phenolic compounds naturally present in virgin olive oils (VOOs) to aid in the understanding of the beneficial effects of the traditional Mediterranean diet. The most important phenolic compounds in VOO are phenolic acids and alcohols, secoiridoids, lignans, and flavonoids (Bendini *et al.*, 2007). The phenol content may be affected by many factors, such as the olive variety, soil, altitude, region, time of harvest and extraction process (Jiménez *et al.*, 2013). It has been demonstrated that the location in which olives are grown has a significant effect on the composition of olive oils of the same cultivar (Ouni *et al.*, 2012). The health benefits of virgin olive oils produced in the same geographical area may vary according to their phenolic compound contents.

Despite the myriad of potential health benefits of olive oil phenolic compounds, little information is available on the potential preventive effects of olive oil phenolic compounds on osteoporosis (Hagiwara *et al.*, 2011; Puel *et al.*, 2007, 2008). In Europe, clear differences are observed in the severity of osteoporosis, the lowest incidence has been reported in the Mediterranean area (Puel *et al.*, 2007). This occurrence has been attributed to a diet rich in olive oil (Fernández-Real *et al.*, 2012, Rivas *et al.*, 2013). It has been shown that the consumption of olives, olive oil, and oleuropein as well as hydroxytyrosol, the main phenolic compounds found in olive leaves and fruit, respectively, can prevent the loss of bone mass in animals (Puel *et al.*, 2007). Moreover, *in vivo* and *in vitro* studies have shown that luteolin, a flavonoid present in VOO, reduces the loss of bone mass in patients with postmenopausal osteoporosis by reducing osteoclast differentiation and function (Lee *et al.*, 2009, Kim *et al.*, 2011). However, the luteolin concentration used in these experiments was higher than those found in virgin olive oil samples.

It should be noted that the *in vivo* biological properties of olive oil phenolic compounds depend on gastrointestinal stability and the extent of absorption and metabolism of the compounds (Mateos *et al.*, 2011). The metabolic fate of phenolic compounds after ingestion has been a key area of research and

investigation concerning the *in vivo* mechanisms by which such compounds are effective (Suarez *et al.*, 2011). In the case of VOO, studies have focused on specific compounds, such as hydroxytyrosol and tyrosol. Visioli *et al.* (2002) identified these compounds in urine, both in conjugated and free forms. Alternatively, the study by Miró-Casas *et al.* (2003) reported the presence of the conjugated forms of hydroxytyrosol in plasma. Moreover, Corona and coworkers (2006) conducted a detailed investigation on the absorption, metabolism and microflora-dependent transformation of the virgin olive oil phenolic compounds hydroxytyrosol, tyrosol and their conjugated forms, such as oleuropein. The conjugated forms underwent rapid hydrolysis under gastric conditions, resulting in significant increases in the amount of free hydroxytyrosol and tyrosol entering the small intestine. In addition, Mateos and coworkers (2011) showed that both hydroxytyrosol acetate and hydroxytyrosol were transferred across human Caco-2/TC7 cell monolayers.

Currently, the most widely used approach in the examination of the potential relationship between VOO phenolic compounds and bone health is based on specific phenolic compounds, such as oleuropein (Santiago-Mora *et al.*, 2011). However, this analysis lacks information regarding the complex interactions among these compounds. It has been established that individual phenolic compounds and phenolic fractions that mainly contained a sole phenolic compound were not equivalent in their abilities to inhibit human breast cancer cell growth (Lozano-Sánchez *et al.*, 2010). Therefore, it is necessary to consider that a combinatorial effect is possible between phenolic compounds and other substances present in phenolic extracts. In this regard, *in vitro* studies should always consider the effect of olive oil active fractions to investigate and promote the development of appropriate recommendations for overall dietary habits.

Accordingly, the first aim of this study was to investigate the influence of the olive variety and the elevation of the orchards on the phenolic compound content of Sicilian virgin olive oils. In addition, because the biological activity of VOO phenols appears to have combinatorial effects, the second aim was to examine the effects of VOO phenolic extracts on osteoblast cell growth using the human MG-63 osteosarcoma cell line.

2. MATERIAL AND METHODS

2.1. Chemicals

Standards of apigenin, luteolin, hydroxytyrosol, tyrosol, vanillin and *p*-coumaric, ferulic, and vanillic acids were purchased from Sigma-Aldrich (St. Louis, MO) and (+)-pinoresinol was purchased from Arbo-Nova (Turku, Finland). Stock solutions of phenolic

compounds were prepared in methanol and stored at -20°C . All of the solvents used were of analytical or HPLC grade (Sigma-Aldrich). The water was of Milli-Q quality (Millipore Corp, Bedford, MA, USA).

2.2. Oil Samples

Sicilian olive samples from eight different cultivars (Biancolilla, Passulunara, Tonda iblea, Nocellara del belice, Nocellara del belice Organic and Nocellara etnea from three different altitudes) were provided by Sicilian oil producers. Olives were hand-picked at the same stage of maturity and the same laboratory mill was used to prepare olive oil samples. Virgin olive oil samples were obtained using an Abencor analyzer (Abengoa S.A., Seville, Spain). This system reproduces the industrial process at a laboratory scale and consists of three basic elements: hammer mill, thermo-beater, and pulp centrifuge (Martínez *et al.*, 1975). The milling of olive fruits was performed using a stainless-steel hammer mill operating at 3000 rpm equipped with a 5 mm sieve. The resulting olive paste was immediately kneaded in a mixer at 50 rpm for 30 min at 30°C . Centrifugation of the kneaded olive paste was performed in a basket centrifuge at 3500 rpm for 1 min. After centrifugation, the oil obtained was decanted and stored in amber glass bottles at 4°C in the dark and without headspace until analysis.

2.3. Phenolic compounds

To isolate the phenolic fraction of olive oils, the method proposed by the International Olive Oil Council (IOOC, 2009) was used. Briefly, the analytical methodology consists of olive oil extraction with methanol/water (80/20), an ultrasonic bath for 15 min at ambient temperature and centrifugation at 5000 rpm for 25 min. After that, an aliquot of the supernatant phase is filtered through a 5 mL plastic syringe using a Millex®-HV PVDF $0.45\ \mu\text{m}$ filter (Millipore Corp, Billerica, MA, USA). The extractions were repeated three times, and phenolic extracts were stored at -20°C until further analysis. The total phenolic content was measured by HPLC using a UV-Vis detector in accordance with IOOC's methodology (IOOC, 2009). Syringic acid was used as the internal standard. HPLC analyses were performed with a Varian ProStar HPLC unit (Walnut Creek, CA, USA) equipped with a Varian 230 UV-Vis detector. The total phenolic content—expressed in $\text{mg}\cdot\text{kg}^{-1}$ of tyrosol—was calculated by measuring the sum of the areas of the related chromatographic peaks, according to the method proposed by the IOOC. Secoiridoid compounds were quantified at 280 nm using the response factors determined by Mateos *et al.* (2011).

When standards were available (apigenin, luteolin, hydroxytyrosol, tyrosol, vanillin, (+)-pinoresinol

and *p*-coumaric, ferulic, and vanillic acids), ultra performance liquid chromatography coupled to time-of-flight mass spectrometry analysis (UPLC-TOF-MS) were performed to detect and quantify individual phenolic compounds in phenolic extracts. The UPLC system consisted of an AcQuity™ UPLC equipped with a binary pump system (Waters, Milford, MA, USA) and an AcQuity UPLC™ BEH C18 column ($1.7\ \mu\text{m}$, $100\ \text{mm}\times 2.1\ \text{mm}$ i.d.) from Waters. The UPLC-TOF-MS methodology was described elsewhere (Jiménez *et al.*, 2013; Rivas *et al.*, 2013). The identification of phenolic compounds was carried out by comparing both the retention times and MS spectra from the olive oil samples and standards when available.

2.4. Cell Culture

The human osteosarcoma cell line MG-63 was purchased from American Type Cultures Collection (ATCC, Manassas, VA) and maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA) with 100 IU/ml penicillin (Lab Roger SA, Barcelona, Spain), $50\ \mu\text{g}\cdot\text{mL}^{-1}$ gentamicin (Braun Medical SA, Jaen, Spain), $2.5\ \mu\text{g}\cdot\text{mL}^{-1}$ amphotericin B (Sigma, St Louis, MO, USA), 1% glutamine (Sigma, St Louis, MO, USA), and 2% HEPES (Sigma, St Louis, MO, USA) and was supplemented with 10% foetal bovine serum (FBS) (Gibco, Paisley, UK). Cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . Cells were detached from the culture flask with a solution of 0.05% Trypsin (Sigma, St Louis, MO, USA) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St Louis, MO, USA) and were then washed and suspended in a complete culture medium with 10% FBS. Prior to the beginning of each experiment, all of the cells were grown in oestrogen-free media (DMEM without red phenol) for at least 24 hours.

2.5. Cell Proliferation Assay

Cell proliferation was determined using the MTT method (Sigma-Aldrich Chemie). This is a colorimetric method that measures the chemical reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into formazan, which is directly proportional to the number of viable cells in the tested sample. Osteoblasts were seeded at 1×10^4 cells $\cdot\text{mL}^{-1}$ per well into a 96-well plate (Falcon Becton Dickinson Labware, New Jersey) in an oestrogen-free culture medium without FBS and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 24 hours. Then, the media was replaced with DMEM containing different concentrations of prepared phenolic extracts dissolved in a fresh culture medium at concentrations of 0.001, 0.0001 and 0.00001%. All of the experiments included cells incubated under

the same conditions without treatment extracts and cells incubated with 0.001% methanol as an internal control. The highest solvent concentration in culture media (0.001%v/v methanol) had no significant effects on cell proliferation. Upon completion of the treatment, the media was replaced with DMEM, without phenol-red, containing $0.5 \text{ mg} \cdot \text{mL}^{-1}$.

MTT (Sigma, St Louis, MO, USA) and was incubated for 4 hours. Cellular reduction of the tetrazolium ring of MTT resulted in the formation of formazan crystals in the form of dark-purple, water-insoluble deposits. After incubation, the media was aspirated and DMSO was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a spectrophotometer (Sunrise TM, Tecan, Männedorf, Switzerland). Cell proliferation percentages (%>control) were calculated compared to cell cultures treated with methanol alone (controls).

2.6. Statistical Analysis

A statistical analysis was performed using SPSS (v. 20.0, Statistical Package for the Social Sciences, Chicago, IL). Numerical data on cell proliferation are expressed as the mean of three independent experiments. Statistically significant differences in the effects on proliferation between the extracts and the control were analyzed by the Student's t-test. The results for phenolic compounds are the average of at least three repetitions. A two-way analysis of variance (ANOVA) was performed to examine the proliferation by considering the concentration, the olive oil variety and the interaction between these two factors. A one-way ANOVA was used to examine phenolic compound concentrations by considering the olive oil variety. The homogeneity between the olive oil variety samples was tested using the Levene's test. Differences between the means were evaluated using the Games-Howell and Bonferroni tests. The significance level in all of the statistical analyses was set at 0.05. The level of significance of differences between the phenolic compound mean values was determined at 5% by a one-way ANOVA using Tukey's test. Principal components analysis (PCA) was applied to the chemical and proliferation data. PCAs were carried out for all raw data processed with a cross-validation method. The data were auto-scaled before analysis.

3. RESULTS AND DISCUSSION

3.1. Identification and quantification of phenolic compounds in VOO

Phenolic compounds are an essential component of VOO quality. The most important phenolic compounds that have been identified in VOO are divided into different groups, such as phenolic acids, phenol alcohols, secoiridoids, lignans, and flavones (Bendini

et al., 2007). In this study, the concentrations of phenolic compounds were determined in olive oil samples that were collected from different Sicilian olive tree cultivars. Table 1 shows the mean values for the total and individual phenolic compounds detected in oils.

The total phenolic content of Sicilian oils was measured by HPLC using a UV-Vis detector in accordance with IOOC's methodology (2009). The differences found in the phenol content among the diverse VOO samples are as expected, as the phenolic content of virgin olive oil is influenced by the type of cultivar, production method and altitude. The oil sample obtained from the Nocellara etnea variety, which is produced at an altitude of 100 m, was the sample with the highest amount of phenolic compounds, while the samples obtained from the Tonda iblea variety contained the lowest. The total phenolic compound contents in the varieties examined in this study were higher than those measured in other studies using spectrophotometry methods (Baccouri *et al.*, 2007; Dugo *et al.*, 2004; Patumi *et al.*, 2003) but were similar to those measured using chromatographic analysis (Saitta *et al.*, 2002).

Significant quantitative differences in the total phenol contents were observed in Nocellara etnea olive oils produced at different altitudes. The highest concentration of phenols was observed in olive oils obtained from plants cultivated at 100 m, and Nocellara etnea olives grown at a higher altitude (900 m) showed the lowest levels of phenolic compounds. This result is contradictory to several reports in which the total phenol content is positively correlated with altitude (Mousa *et al.*, 1996; Ouni *et al.*, 2012).

The phenol alcohols found in the olive oils studied were hydroxytyrosol and tyrosol. The concentration of hydroxytyrosol ($4.26\text{--}9.93 \text{ mg} \cdot \text{kg}^{-1}$) was generally higher than that of tyrosol ($2.12\text{--}4.53 \text{ mg} \cdot \text{kg}^{-1}$) and was similar to that reported by other authors (Baccouri *et al.*, 2007). The highest concentrations of these compounds were observed in Nocellara del belice olive oils. In this variety, the mean values of hydroxytyrosol were higher in olive oils obtained from conventionally cultivated olive trees than in those obtained from organic cultivation. Nocellara etnea olive oils from olives grown at a higher altitude have greater tyrosol and hydroxytyrosol concentrations than Nocellara etnea oils obtained from olives grown at an altitude of 0 m.

The most abundant phenolic compounds found in the VOOs under study were secoiridoids. The concentrations of these compounds ranged from 0.3 to $54.31 \text{ mg} \cdot \text{kg}^{-1}$. In general, these results agree with those found by other authors in the Nocellara del Belice and Biancolilla olive oil varieties (Baccouri *et al.*, 2007). However, the secoiridoid concentrations in Tonda iblea olive oil were lower than those previously reported (Napolitano *et al.*, 2008). In general, the secoiridoid content of Nocellara del belice olive oils obtained from conventional cultivation is

TABLE 1. Phenolic compound contents ($\mu\text{g}\cdot\text{kg}^{-1}$) in Sicilian virgin olive oils

	Biancolilla	Passulunara	Tonda iblea	N. del belice	Organic N. del belice	N. etnea 0 m	N. etnea 100 m	N. etnea 900 m
Phenol alcohols								
Hydroxytyrosol	4.26 \pm 0.12 ^e	8.03 \pm 0.23 ^c	8.01 \pm 0.31 ^c	9.93 \pm 0.18 ^a	8.93 \pm 0.15 ^b	4.35 \pm 0.09 ^e	6.35 \pm 0.25 ^d	6.11 \pm 0.14 ^d
Tyrosol	3.90 \pm 0.11 ^e	4.06 \pm 0.18 ^{bc}	3.32 \pm 0.10 ^d	4.53 \pm 0.15 ^a	4.24 \pm 0.08 ^b	2.12 \pm 0.03 ^f	3.27 \pm 0.01 ^d	2.53 \pm 0.05 ^e
Secoiridoids								
Decarboxymethyl oleuropein aglycone, oxidized dialdehyde form	34.40 \pm 2.81 ^a	22.20 \pm 3.42 ^b	12.39 \pm 1.20 ^e	21.93 \pm 2.18 ^b	20.98 \pm 1.97 ^b	31.43 \pm 4.05 ^a	35.68 \pm 2.12 ^a	11.46 \pm 1.34 ^c
Decarboxymethyl oleuropein aglycone, dialdehyde form	18.49 \pm 1.26 ^a	3.24 \pm 1.31 ^b	0.47 \pm 0.09 ^e	0.52 \pm 0.01 ^d	0.43 \pm 0.05 ^e	0.60 \pm 0.02 ^c	0.30 \pm 0.01 ^f	0.50 \pm 0.012 ^d
Oleuropein aglycone dialdehyde form	1.98 \pm 0.02 ^c	0.23 \pm 0.01 ^e	0.54 \pm 0.04 ^d	0.59 \pm 0.02 ^d	0.62 \pm 0.07 ^d	2.72 \pm 0.01 ^b	9.78 \pm 0.08 ^a	0.50 \pm 0.02 ^d
Decarboxymethyl ligstroside aglycone, oxidized dialdehyde form	41.55 \pm 3.42 ^b	20.32 \pm 1.78 ^e	17.34 \pm 2.89 ^f	36.03 \pm 2.14 ^c	35.97 \pm 2.17 ^c	30.01 \pm 1.52 ^d	52.06 \pm 2.89 ^a	17.90 \pm 1.67 ^f
Decarboxymethyl ligstroside aglycone, dialdehyde form	52.09 \pm 4.31 ^a	18.33 \pm 1.89 ^{bc}	12.32 \pm 2.49 ^d	19.23 \pm 1.65 ^b	17.84 \pm 1.24 ^c	19.78 \pm 2.43 ^b	54.31 \pm 1.83 ^a	5.49 \pm 0.35 ^e
Ligstroside aglycone, dialdehyde form	4.58 \pm 0.21 ^a	2.34 \pm 0.12 ^b	0.79 \pm 0.15 ^d	0.91 \pm 0.08 ^d	0.82 \pm 0.05 ^d	1.95 \pm 0.03 ^c	4.81 \pm 0.35 ^a	1.03 \pm 0.24 ^c
Oleuropein aglycone, oxidized aldehyde and hydroxylic form	7.26 \pm 0.08 ^b	10.23 \pm 0.31 ^a	4.25 \pm 0.83 ^c	3.81 \pm 0.04 ^{cd}	2.39 \pm 0.14 ^e	2.89 \pm 0.01 ^e	3.58 \pm 0.043 ^d	3.78 \pm 0.04 ^d
Oleuropein aglycone, aldehyde and hydroxylic form	19.49 \pm 0.21 ^b	14.52 \pm 0.32 ^d	5.39 \pm 0.03 ^e	15.98 \pm 0.11 ^c	16.03 \pm 0.14 ^c	15.32 \pm 0.02 ^{cd}	54.31 \pm 0.05 ^a	1.98 \pm 0.09 ^f
Ligstroside aglycone, oxidized aldehyde and hydroxylic form	4.02 \pm 0.01 ^c	1.64 \pm 0.05 ^d	0.25 \pm 0.03 ^e	8.31 \pm 0.17 ^b	8.65 \pm 0.12 ^b	9.51 \pm 0.21 ^b	21.91 \pm 0.52 ^a	4.95 \pm 0.05 ^c
Ligstroside aglycone, aldehyde and hydroxylic form	8.12 \pm 0.41 ^b	2.56 \pm 0.12 ^d	2.32 \pm 0.15 ^d	2.54 \pm 0.13 ^d	1.99 \pm 0.04 ^e	4.94 \pm 0.31 ^c	13.61 \pm 0.21 ^a	8.60 \pm 0.12 ^b
Phenolic acids								
Vanillic acid	0.15 \pm 0.01 ^a	nd	0.12 \pm 0.01 ^c	0.13 \pm 0.09 ^{bc}	nd	0.12 \pm 0.02 ^c	0.13 \pm 0.01 ^{bc}	0.14 \pm 0.01 ^a
Vanillin	0.16 \pm 0.01 ^a	nd	0.01 \pm 0.01 ^c	0.03 \pm 0.01 ^{bc}	0.01 \pm 0.01 ^c	0.01 \pm 0.01 ^c	0.01 \pm 0.01 ^c	0.01 \pm 0.01 ^c
p-coumaric acid	0.43 \pm 0.01 ^a	0.32 \pm 0.02 ^b	0.15 \pm 0.02 ^d	0.15 \pm 0.01 ^d	0.18 \pm 0.03 ^c	0.16 \pm 0.05 ^{cd}	0.20 \pm 0.04 ^{bc}	0.10 \pm 0.006 ^e
Ferulic acid	0.72 \pm 0.02 ^c	0.34 \pm 0.02 ^e	0.65 \pm 0.03 ^{cd}	0.62 \pm 0.02 ^{cd}	0.55 \pm 0.02 ^d	1.23 \pm 0.06 ^b	0.59 \pm 0.01 ^d	3.21 \pm 0.34 ^a
Cinnamic acid	0.01 \pm 0.004 ^e	0.02 \pm 0.004 ^e	0.01 \pm 0.001 ^c	0.01 \pm 0.009 ^c	0.01 \pm 0.002 ^e	0.05 \pm 0.001 ^b	0.06 \pm 0.003 ^b	0.31 \pm 0.001 ^a
Flavonoids								
Luteolin	3.74 \pm 0.51 ^{a,b}	2.23 \pm 0.21 ^c	1.54 \pm 0.06 ^d	1.85 \pm 0.04 ^d	1.54 \pm 0.06 ^d	4.32 \pm 0.95 ^a	3.29 \pm 0.04 ^b	2.24 \pm 0.21 ^c
Apigenin	1.61 \pm 0.03 ^b	1.65 \pm 0.06 ^b	1.37 \pm 0.05 ^c	1.23 \pm 0.01 ^{cd}	1.37 \pm 0.09 ^c	1.96 \pm 0.06 ^a	1.03 \pm 0.02 ^d	0.84 \pm 0.003 ^e
Lignan								
Pinoresinol	0.40 \pm 0.02 ^b	0.50 \pm 0.035 ^b	0.14 \pm 0.017 ^d	0.13 \pm 0.04 ^d	0.15 \pm 0.07 ^d	0.23 \pm 0.02 ^c	0.21 \pm 0.09 ^c	1.49 \pm 0.05 ^a
Total phenols	321.07 \pm 12.57 ^d	290.23 \pm 5.92 ^e	204.71 \pm 2.89 ^g	385.32 \pm 9.29 ^e	324.32 \pm 7.36 ^d	553.24 \pm 6.38 ^b	638.00 \pm 12.64 ^a	213.45 \pm 9.75 ^f

The values are expressed as the mean for the three analyses. Tukey's test has been used to assess significance. Different letters in the same row indicate significant differences ($p < 0.05$). nd: not detected N, Nocellara.

higher than that found in oils obtained from organic cultivation. There were significant differences in the secoiridoid content among Nocellara etnea oils obtained from olives cultivated at different altitudes. Nocellara etnea olives grown at 100 m produced oil with the highest content of secoiridoids, and the content decreased in oils obtained from olives grown at a higher altitude (900 m).

Phenolic acids were the first group of phenolic compounds to be identified in virgin olive oil (Servili *et al.*, 2009). In recent years, phenolic acids have received considerable attention as they are essential to olive oil quality and nutritional properties. The phenolic acids observed in the VOOs in this study were *p*-coumaric and ferulic acids. Concentrations of ferulic acid ranged from 0.34 to 3.21 mg·kg⁻¹. In all the samples investigated, *p*-coumaric acid concentrations did not exceed 0.43 mg·kg⁻¹. Vanillic acid was not detected in Passulunara and Nocellara del belice organic olive oils. These results agree with those reported by other authors (Saitta *et al.*, 2002). With regard to phenolic acid concentrations in the Novellara etnea variety at different altitudes, the highest *p*-coumaric acid concentration was found at 100 m (0.20 µg·kg⁻¹), while cinnamic acid and ferulic acid concentrations were higher in oils from N. etnea grown at 900 m.

Flavonoids and lignan groups of phenolic compounds abundant in plants are known to have many beneficial biological effects, including anti-inflammatory, antioxidant and oestrogenic activities (Adlercreutz, 2007). Considering flavone concentrations, luteolin and apigenin were more abundant in the Biancolilla and Nocellara etnea varieties. Luteolin concentrations ranged from 1.54 to 4.32 mg·kg⁻¹ and were higher than apigenin concentrations (0.84–1.96 mg·kg⁻¹). Nocellara etnea oils obtained from olive groves at an altitude of 0 m had higher flavonoid concentrations than those obtained from groves at a higher altitude. Concentrations of the lignan pinoresinol were within the range of 0.13–1.49 µg·kg⁻¹. Olive oils obtained from Nocellara etnea olive groves at an altitude of 900 m had higher pinoresinol concentrations than those obtained from groves at a lower altitude.

3.2. Effect of olive oil phenolic compound extracts on MG-63 osteoblast cell proliferation

Nutritional and pharmacological therapies are needed to aid in the prevention of bone loss due to aging. In this study, the potential effects of VOO phenolic extracts obtained from Sicilian monovarietal olive oils on the proliferation of human osteosarcoma cells were investigated. MG-63 osteoblast cells were cultured in the absence and presence of phenolic extract dilutions in a fresh culture medium. The highest solvent concentration in the culture media (0.001%, v/v methanol) had no significant effects on cell proliferation.

The hypothesis of this study was that the phenolic fractions of each VOO variety would have differing effects on the induction of osteoblast proliferation. MG-63 cells were treated with various concentrations of extracts to determine the biological activities of the complex multi-component phenolic extracts. All extracts, with the exception of Tonda iblea, stimulated MG-63 cell growth significantly in a non-concentration-dependent manner, with the following order of potency: Nocellara del belice>Biancolilla>Organic Nocellara del belice>Nocellara etnea 0 m>Nocellara etnea 100 m>Nocellara etnea 900 m>Passulunara>Tonda iblea. Treatment of osteoblast cells with olive oil phenolic extracts increased the number of cells by 13.77–30.98% compared with controls (Table 2).

Differences in the ability of individual phenolic extracts to stimulate osteoblast proliferation were observed (Table 2). For instance, concentrations of 0.001% and 0.0001% were needed to increase cell proliferation significantly when MG-63 cells were cultured in the presence of Passulunara and Nocellara etnea at 900 m phenolic extracts. On the contrary, the olive oil phenolic extracts of Biancolilla, Nocellara del Belice, Nocellara etnea at 0 and 100 m altitudes significantly increased the MG-63 cell proliferation at the lowest concentration.

The VOO phenolic extracts with the highest activity were those obtained from the Nocellara del belice variety, as they significantly increased cell proliferation by 30.98%. In addition, treatment with organic Nocellara del belice VOO phenolic extracts increased cell proliferation by 28.46% at the lowest concentration. However, Tonda iblea phenolic extracts did not stimulate cell proliferation.

Although the impact of olive oil phenolic extracts on MG-63 cells is well-known, the mechanism by which they induce MG-63 cell proliferation is not fully understood. In this regard, certain observations may help describe this mechanism. Oxidative stress, which increases the level of intracellular reactive oxygen species, has been reported to inhibit bone metabolism (Hagiwara *et al.*, 2011). Olive oil phenolic compounds have strong antioxidant properties (Visioli y Bernardini, 2011), and hydroxytyrosol has been shown to decrease H₂O₂ levels in MC3T3-E1 osteoblast cells (Hagiwara *et al.*, 2011). In addition, it has been demonstrated that H₂O₂ suppresses differentiation markers (such as alkaline phosphatase activity), osteoblastic cell mineralization and type I collagen gene expression (Hosoya *et al.*, 1998).

Even though the phenolic contents differed among the different VOOs studied, most phenolic extracts increased cell proliferation significantly. It should be noted that the effect on osteoblast proliferation was observed in a non-concentration-dependent manner. The oils obtained from the

TABLE 2. Effect of Sicilian VOO phenolic extracts on MG-63 cell growth as determined by MTT. Cells were treated with various concentrations of VOO phenolic extracts or vehicle alone (control) for 24 h

Treatment	Concentration (% v/v)	Proliferation (% control) ^a	t	p
Control		100%		
Biancolilla	0.001	127.76±11.66	-3.471	0.002
	0.0001	128.72±4.96	-3.719	0.001
	0.00001	127.59±8.45	-3.519	0.001
Passulunara	0.001	124.49±5.04	-3.171	0.003
	0.0001	121.24±6.87	-2.730	0.010
	0.00001	115.34±11.92	-1.618	0.116
Tonda iblea	0.001	110.96±19.53	-1.073	0.295
	0.0001	102.17±6.21	-0.731	0.473
	0.00001	101.57±6.42	-0.523	0.607
Nocellara del belice	0.001	130.98±2.00	-4.039	0.0001
	0.0001	123.65±8.81	-3.010	0.005
	0.00001	119.00±11.05	-2.385	0.024
Organic Nocellara del belice	0.001	127.73±3.78	-3.603	0.001
	0.0001	124.02±2.78	-3.127	0.004
	0.00001	128.46±2.08	-3.710	0.001
Nocellara etnea 0 m	0.001	126.01±7.47	-8.513	0.0001
	0.0001	125.49±6.81	-8.716	0.0001
	0.00001	113.77±13.99	-3.004	0.007
Nocellara etnea 100 m	0.001	125.02±6.31	-8.842	0.0001
	0.0001	121.054±6.70	-6.873	0.0001
	0.00001	116.40±12.46	-4.446	0.0001
Nocellara etnea 900 m	0.001	125.27±15.97	-4.960	0.0001
	0.0001	118.39±16.25	-3.559	0.002
	0.00001	106.43±10.29	-1.030	0.314

^a The values are the mean±SD (%) from three separate experiments performed in triplicate. Statistically significant differences were analyzed by Student's t-test.

Tonda iblea variety did not have any effect on osteoblast cell proliferation, and it has been noted that this variety has a low total phenol content. However, there are no major qualitative differences in the concentration of phenolic compounds between the Tonda iblea variety and other varieties studied. Passulunara and Nocellara etnea varieties grown at 900 m induce a significant increase in cell proliferation at a phenol extract concentration of 0.001%, even though they are varieties with low total phenolic contents. In addition, phenol extracts from oils obtained from the Nocellara etnea variety grown at 100m, which is the variety with the highest total phenol content, did not exert the greatest effect on osteoblast cell proliferation. These data suggest that other substances present in this extract may be

responsible for or influence the effect on MG-63 cell proliferation. Further studies are required to identify other bioactive substances and the mechanisms by which VOO phenolic extracts induce MG-63 cell proliferation.

The mean dietary intake of virgin olive oil phenols has been estimated to be approximately $68.5 \pm 104.0 \text{ mg} \cdot \text{day}^{-1}$ in a Mediterranean population, which represented approximately 11% of the total phenolic compound intake (Treserra-Rimbau *et al.*, 2013). Considering these amounts, the concentration of phenols used in this study are easily achieved with the actual daily intake of virgin olive oil and are potentially reached in the intestine after the consumption of olive oil. However, we should acknowledge that *in vitro* studies on biocompounds should always consider intestinal absorption and biotransformation. In this regard, research on humans (Miró-Casas *et al.*, 2002; Visioli *et al.*, 2003) and animals (Tuck *et al.*, 2002) has shown that the dominant virgin olive oil phenols, hydroxytyrosol and tyrosol, are bioavailable and, as such, a fraction of the oral dose can be recovered in the urine, with >90% of the urinary excretion in the form of metabolites, which are mainly O-glucuronoided conjugates. In addition, after virgin olive oil administration, O-methylated forms, such as homovanillyl alcohol or homovanillyl acid, and monosulphated conjugates have been identified in plasma and urine (Mateos *et al.*, 2011; Miró-Casas *et al.*, 2002; Visioli *et al.*, 2003). A further study found that the absorption of administered ligstroside-aglycone, hydroxytyrosol, tyrosol and oleuropein aglycone was as high as 55–66% of the ingested phenols in humans, indicating an effective intestinal absorption of these compounds (Visiers *et al.*, 2002). Unfortunately, the knowledge available on the metabolic fate of VOO-derived complex phenols is still scarce (Lozano-Sánchez *et al.*, 2010), hence the need for research on the plasma and urine concentrations of the free forms of various secoiridoid aglycones.

Although these experimental studies support the hypothesis that VOO phenolic compounds exert a beneficial effect on osteoblasts further studies assessing the *in vivo* accessibility of VOO phenolic compounds to osteoblast cells should be carried out. Accordingly, as the metabolism of VOO phenolic compounds is not well understood, it is necessary to perform further *in vitro* studies on the intestinal absorption and biotransformation of such compounds.

3.3. Statistical Analysis

As shown in Table 3, an ANOVA demonstrated that MG-63 cell proliferation is influenced by the variety and concentration of olive oil ($p=0.0001$ and $p=0.001$, respectively). The adjusted R-squared value was 0.802, indicating

TABLE 3. Results of an analysis of variance for MG-63 cell growth as a function of the olive oil variety and concentration and for phenol concentrations as a function of the olive oil variety

Source	Sum of squares	Degrees of freedom	Mean square	F Statistic	P value	Adjusted R squared
MG-63 proliferation¹						
Corrected Model	1417.5	9	157.5	11.3	0.0001	0.802
Intercept	348367.9	1	348367.9	25046.9	0.0001	
Concentration	303.4	2	151.7	10.9	0.001	
Olive oil variety	1114.1	7	159.1	11.4	0.0001	
Error	194.7	14	13.9			
Total	349980.1	24				
Corrected Total	1612.2	23				
Phenol alcohols²						
Inter-group	128.256	7	18.322	9.842	0.0001	0.811
Intra-group	29.785	16	1.862			
Total	158.041	23				
Secoiridoids²						
Inter-group	99072.4	7	14153.2	119.142	0.0001	0.981
Intra-group	1900.6	16	118.792			
Total	100973.0	23				
Phenolic acids²						
Inter-group	21.211	7	3.030	728.43	0.0001	0.996
Intra-group	0.067	16	0.004			
Total	21.278	23				
Flavonoids²						
Inter-group	31.385	7	4.484	32.282	0.0001	0.933
Intra-group	2.222	16	0.139			
Total	33.607	23				
Lignans²						
Inter-group	3.237	7	0.462	553.930	0.0001	0.995
Intra-group	0.013	16	0.001			
Total	3.251	23				
Total phenolic compounds²						
Inter-groups	492390.1	7	70341.4	1106.1	0.0001	0.997
Intra-groups	1017.4	16	63.5			
Total	493407.6	23				

¹ Two-way ANOVA; ² One-way ANOVA.

that olive oil variety and phenol concentrations explain 80.2% of variability in the proliferation. The Bonferroni test showed that the Tonda iblea variety had different effects on MG-63 proliferation compared to the rest of the olive oil varieties studied. In addition, it was demonstrated that there are statistically significant differences ($p=0.001$) between the effects of the 0.001% and 0.00001% sample concentrations on MG-63 proliferation. On the contrary, there are no significant differences in the effect of the 0.001% concentrations on cell proliferation with respect to the other concentrations studied ($p>0.005$). An ANOVA of the phenol

concentration revealed the influence of the variety of olive oil in all the groups of compounds studied ($p=0.0001$). The statistical test allowed us to group the eight varieties of olive oil into five categories according to their phenol content: I (Tonda iblea and Nocellara etnea 900 m), II (Biancolilla, Passulunara and Organic Nocellara del belice), III (Nocellara del belice), IV (Nocellara Etnea 0 m) and V (Nocellara Etnea 100 m).

Multivariate PCA was used to further describe the differences observed in the VOO samples obtained from different cultivars and grove locations. The score and loading plot obtained by PCA

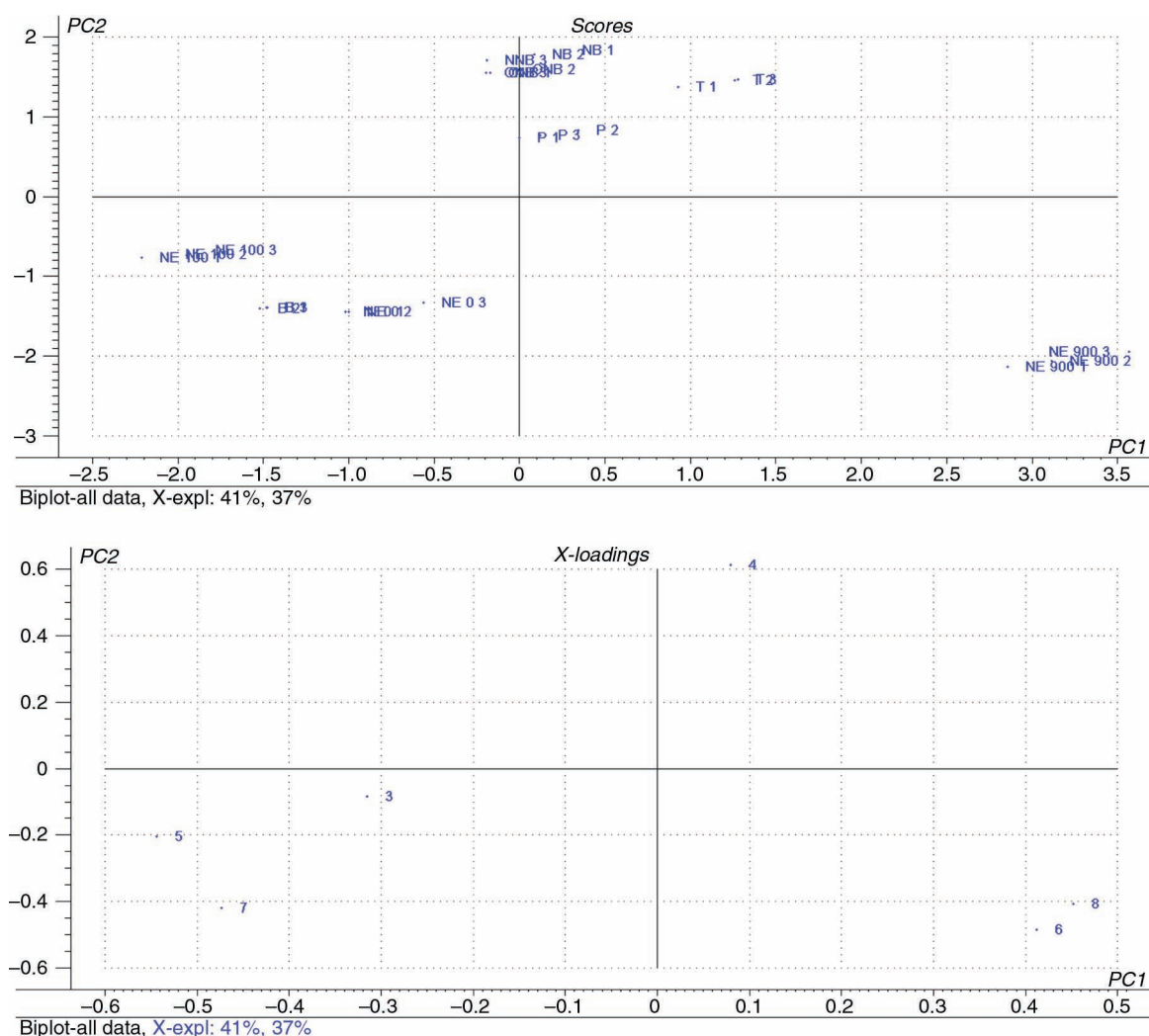


FIGURE 1. Bi-Plot (A. Scores and B. Loadings) of the principal component analysis model for the Sicilian VOO. Samples are plotted by selected letters, Biancolilla (B), Passulunara (P), Tonda iblea (T), Nocellara del belice (NB), Organic Nocellara del belice (ONB), and Nocellara etnea (NE), and by numbers, 1, 2 and 3, corresponding to the 0.001, 0.0001 and 0.0001% extract concentration in osteoblast cell culture medium.

based on the phenol concentration values and the effect of the VOOs on cell proliferation are shown in Figure 1. Across the first-dimension PC1 (accounting for 41% of the total variance), the type of cultivar and the grove location were discriminated, whereas across the second dimension (accounting for 37% of the total variance), the cultivation method (organic or conventional for Nocellara del belice) and phenolic extract concentrations in osteoblast cells were discriminated. Table 4 shows the loading of the variables for the Principal Components 1 and 2. Secoiridoids and flavonoids showed the highest loading in Component 1 and phenolic acids and alcohols in Component 2.

Comparisons between the two PCA plots (score and loading) indicated that the variables flavonoids,

phenolic acids and lignans were mainly responsible for the discrimination of the Nocellara etnea olive oils obtained at 900 m. This cultivar, along with Tonda iblea, obtained the highest scores on Component 1, and Nocellara del Belice (organic and conventional) obtained the highest scores on Component 2.

To characterize olive oils from the Nocellara etnea varieties at different altitudes, a multivariate analysis was applied to the chemical data and to the effect of this olive oil variety on osteoblast cell proliferation. PCA discriminated the samples by altitude (Figure 2; Table 5), thus highlighting the important role of the environment in determining the phenolic profile and nutritional properties of virgin olive oil (Servili *et al.*, 2009). Comparisons

TABLE 4. X Loading of the variables included in Principal Components 1 and 2 of Sicilian VOO

	Variable	PC1	PC2
1	MG-63 Proliferation	-0.32	-0.08
2	Phenol alcohols	0.08	0.61
3	Secoiridoids	-0.54	-0.20
4	Phenolic acids	0.41	-0.48
5	Flavonoids	-0.47	-0.42
6	Lignans	0.45	-0.41

between the two PCA plots (score and loading) indicated that the variables lignans and phenolic acids were mainly responsible for the discrimination of

the oils at 900 m of altitude, whereas the variables secoiridoids were observed at 100 m of altitude for the oils. Finally, osteoblast cell proliferation and flavonoids were major contributors to the separation of *Nocellara etnea* oils at the lowest altitude.

4. CONCLUSION

In this study, the olive oil phenolic content and its effect on human osteosarcoma cell proliferation showed consistent variability according to the type of cultivar and the grove altitude, even within the same type of cultivar. This behavior demonstrated that the cultivar and the grove location can significantly affect the chemical and health-promoting characteristics of virgin olive oil. Although these

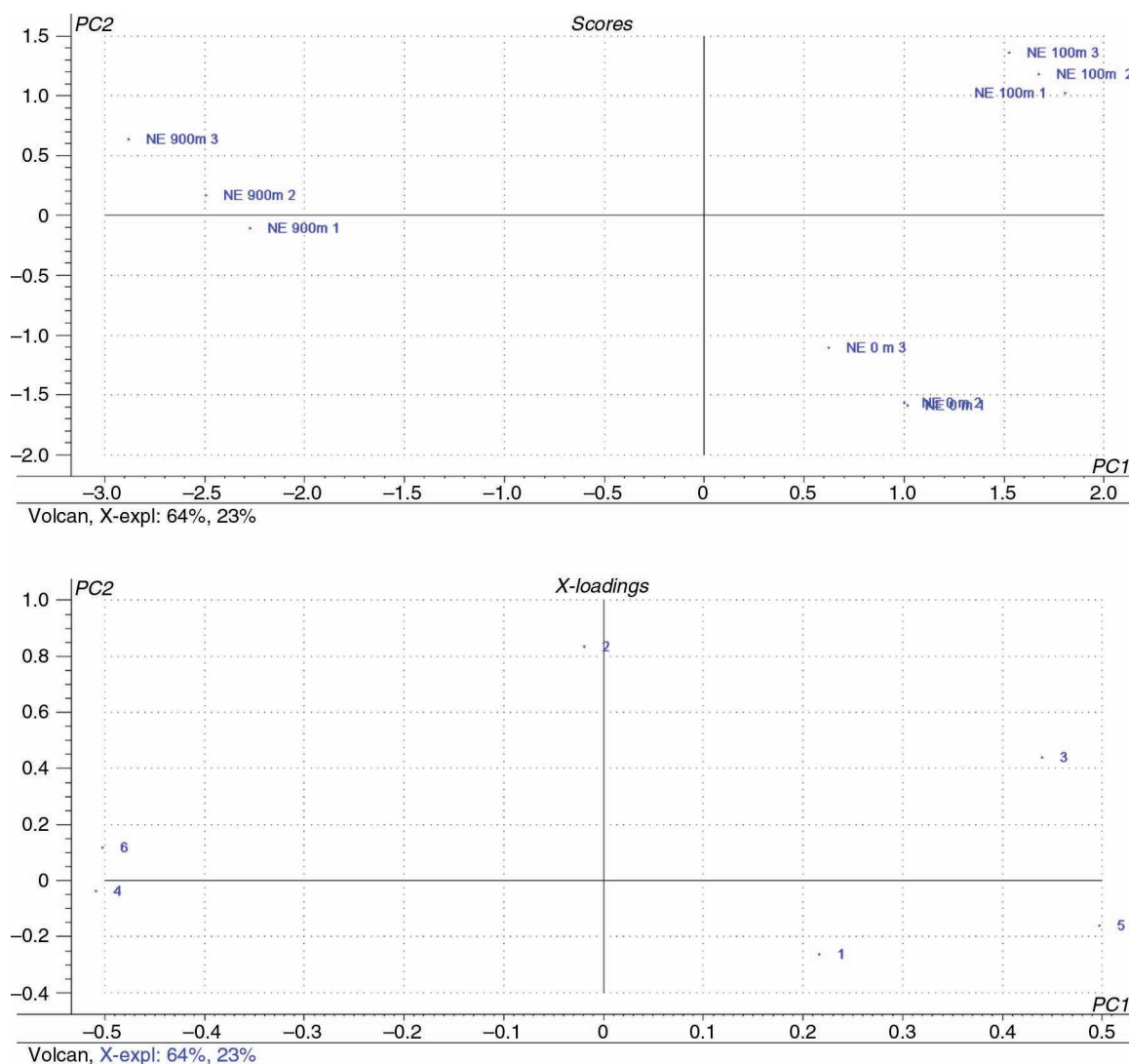


FIGURE 2. Bi-Plot (A. Scores and B. Loadings) of the principal component analysis model for *Nocellara etnea* (NE) VOO at three different altitudes. Samples are plotted by numbers, 1, 2 and 3, corresponding to the 0.001, 0.0001 and 0.0001% extract concentration in osteoblast culture medium.

TABLE 5. X Loading of the variables included in Principal Components 1 and 2 of Nocellara etnea VOO

	Variable	PC1	PC2
1	MG-63 Proliferation	0.21	-0.26
2	Phenol alcohols	-0.001	0.83
3	Secoiridoids	0.44	0.43
4	Phenolic acids	-0.50	-0.003
5	Flavonoids	0.498	-0.16
6	Lignans	-0.50	0.11

experimental studies support the hypothesis that VOO phenolic fractions exert a beneficial effect on bone health, further studies assessing the *in vivo* accessibility of VOO phenolic compounds to osteoblast cells should be performed.

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